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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:	WO 98/00562
C12Q 1/68, C12N 15/10	A1	(43) International Publication Date:	8 January 1998 (08.01.98)

(21) International Application Number: PCT/GB97/01764

(22) International Filing Date: 30 June 1997 (30.06.97)

(30) Priority Data:
9613980.3 2 July 1996 (02.07.96) GB

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(81) Designated States: CA, JP, KR, SG, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DENATURATION OF DOUBLE-STRANDED NUCLEIC ACID

(57) Abstract

Double-stranded nucleic acid is denatured by subjecting a solution thereof to a voltage applied between electrodes spaced by no more than 1.5 mm in a time not previously achievable in electrochemical denaturation. PCR is practised isothermally by periodic application of voltage to produce denaturation. Electrochemical cells and kits for use in the process are provided.

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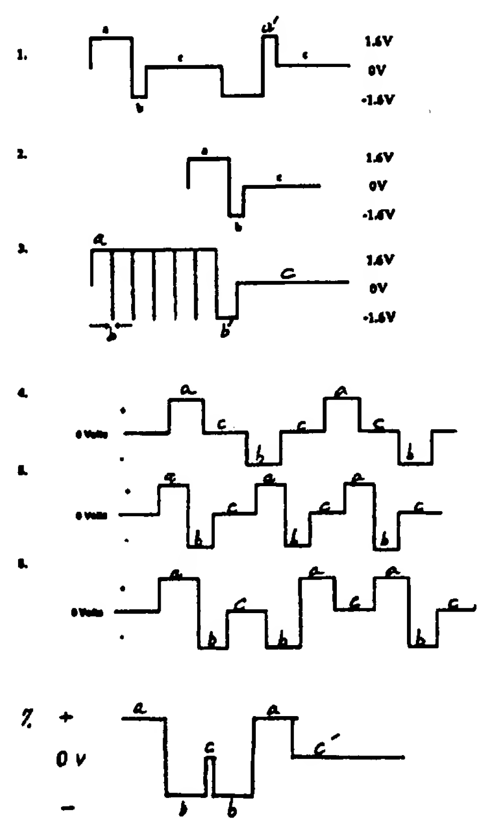


DENATURATION OF DOUBLE-STRANDED NUCLEIC ACID

This invention relates to processes for the treatment of nucleic acid material in order to effect a complete or partial change from double-stranded form to single-stranded form and to processes of amplifying or detecting nucleic acids involving such denaturation processes.

Double-stranded DNA (deoxyribonucleic acid) and DNA/RNA (ribonucleic acid) and RNA/RNA complexes in the familiar double helical configuration are stable molecules that, in vitro, require aggressive conditions to separate the complementary strands of the nucleic acid. Known methods that are commonly employed for strand separation require the use of high temperatures of at least 60°C and often 100°C or use an alkaline pH of 11 or higher. Other methods include the use of helicase enzymes such as Rep protein of E.coli that can catalyse the unwinding of the DNA in an unknown way, or binding proteins such as 32-protein of E.coli phage T4 that act to stabilise the single-stranded form of DNA. The denatured single stranded DNA produced by the known processes of heat or alkali treatment is used commonly for hybridisation studies or is subjected to amplification cycles.

Such separation is a prerequisite of a number of protocols involving the in vitro manipulation of nucleic acids, one example of which is a reaction that produces multiple copies of target sequences of DNA and which employs a heat-stable polymerase enzyme (US Patent No. 4683202, K.B. Mullis et al). This development, known as the polymerase chain reaction (PCR), is of significant commercial importance and strand separation is normally effected by heating the sample to approximately 95°C. The removal of the need to heat the sample would provide a number of benefits. For example, it allows the design of compact and readily controllable apparatus, and the use of higher fidelity mesophyllic enzymes.

WO 92/04470 discloses a process whereby nucleic acid strands are separated by the application of an electric field. The advantages of the electrical method are discussed in greater detail, along with the method's application in amplification reactions such as PCR and ligase chain reaction. Forms of electrochemical cells for carrying out the reaction are described and also the use of "promoter" compounds that enhance the efficiency of denaturation.

Prior to WO92/04470, a number of other workers had described denaturation of DNA in electrochemical cells. However, in none of these cases was single-stranded product left free in solution in useful quantities. Rather, DNA appears to have become irreversibly bound to the surface of the electrode, in which condition it is not available for further participation in processes such as PCR. In the method of electrical denaturation described in WO92/04470, single strands accumulate in solution and their utility and integrity is confirmed by subsequently performing PCR.

In WO92/04470 electrical denaturation of DNA was carried out using an electrode comprising a central rod of glassy carbon encased in a teflon sleeve except at its end. The working electrode was of platinum mesh lying against the teflon sleeve. A calomel reference electrode was used, situated in a side chamber which was connected to the main cell by a capillary tube (see Stanley C.J. et al, J. Immunol. Meth. [1988], 112, 153-161). Using this apparatus the most rapid denaturation was achieved in 15 minutes with the working electrode at a potential of -1V with respect to the reference. The presence of NaCl in the reaction delayed denaturation.

In W092/04470, a PCR reaction is conducted in which there are repeated denaturation operations conducted using the electro-chemical cell described with intervening amplification stages. The denaturation stages are each conducted for a period of five minutes or longer and the total time for the PCR reaction is therefore very extended. Furthermore, the conditions under which the PCR reaction was conducted in

WO92/00470 differ from those of the conventional PCR process in that it was not found possible to use a conventional PCR buffer system. In order to obtain denaturation, it was necessary to conduct the process at a much lower ionic strength than would be consistent with such a buffer system. Excluding the promoter methyl viologen, the process was basically conducted in distilled water.

In WO95/25177 we showed it is possible to conduct a denaturation electrochemically considerably faster than is disclosed in WO92/04470 and to conduct an amplification procedure much faster than is disclosed there.

Although the spacing between the two working electrodes in WO92/04470 is not explicitly stated, it was in fact several millimetres.

An improved method is described in WO95/25177 in which a solution containing said nucleic acid is subjected to a voltage applied between electrodes which approach to within 1.5 mm of one another in said solution. This results in a substantial increase in the rate of denaturation such that WO95/25177 contains examples in which complete denaturation of DNA is achieved within 1 to 2 minutes in comparison with denaturation times of at 25 minutes using the electrode set up of WO92/04470.

It is indicated in WO95/25177 that rather than simply turning the electrical field on and off when conducting PCR using the apparatus described there, one may optionally reverse the field. In WO95/25177, this reversal of the field is seen as being merely an equivalent to turning the field off.

It has now been found surprisingly that the process of electrochemical denaturation and PCR processes based upon it are greatly further accelerated if one employs not only periods during which the electric field is reduced substantially to zero but also further periods during which the field is reversed. The combination of all three field conditions in any order in a cyclic manner in a PCR process

leads to substantially more rapid amplification and produces conditions favourable for the use of mesophyllic polymerases.

Accordingly, the present invention provides a process for denaturing double-stranded nucleic acid which comprises subjecting a solution containing said nucleic acid to a voltage applied between electrodes under conditions such as to convert at least a portion of said nucleic acid to a wholly or partially single-stranded form, wherein said voltage is applied such that there are, in any order, periods of application of voltage with a first polarity, periods of application of voltage with the opposite polarity to said first polarity and periods of substantially reduced applied voltage.

It will be understood that the nucleic acid does not have to be in solution, but may be immobilised by one or both strands to a solid phase immersed in a liquid, e.g. a salt or buffer solution.

The invention also includes a process for amplifying a target sequence of nucleic acid comprising cycles of hybridisation, replication and denaturation of nucleic acid wherein said denaturation is conducted by subjecting a said nucleic acid to a voltage applied between electrodes such as to convert at least a portion of said nucleic acid to a wholly or partially single-stranded form, said voltage being applied such that there are, in any order, periods of application of voltage with a first polarity, periods of application of voltage with the opposite polarity to said first polarity and periods of substantially zero applied voltage.

The reduced voltage is preferably constant and should normally be less than one volt and preferably is less than 0.5V, e.g. less than 0.2V. Most preferably it is substantially zero.

Preferably, the cycles are from 1 second to 5 minutes in length. Preferably the periods during which the voltage is applied with a first polarity and the periods during which the voltage is applied with a second polarity are each somewhat shorter than the periods during which there is substantially no voltage applied. Suitably, the periods during which the

voltage is applied with the first polarity or the opposite polarity are each independently from less than 0.1 seconds to 1 minute. Preferably, the periods of substantially zero applied voltage are each independently from 0.5 seconds to 3 minutes.

Preferably, there is a long positive voltage application, e.g. 5 to 30 seconds, followed by a short negative voltage application 0.1 to 10 seconds, followed by a longer period of substantially zero volts.

The apparatus employed is preferably as described in WO95/25177 or as illustrated herein. Preferably, the electrodes approach to within 1.5 mm of one another or more closely, e.g. within 1 mm or more preferably 0.5 mm of one another. Ideally, the electrodes are adjusted to be spaced by as little as possible whilst ensuring that they do not contact one another to produce a short circuit.

It is preferred to apply a voltage difference of from 0.5 to 3 volts between the electrodes. Voltage differences above 3 volts seem to inhibit denaturation or produce degradation according to the nature of the electrode set up, although the mechanism involved here is presently unknown.

Preferably, the process is conducted at a voltage of 1 to 2 volts, e.g. 1.4 to 1.8 volts measured as a voltage difference between the electrodes.

Preferably, if one of the electrodes is in the nature of a rod, where the electrodes most closely approach one another, one or both of the electrodes is pointed. Such an electrode may be provided with a single point or a plurality of points. There appears to be some inter-relationship between the ideal voltage applied and the shape of the electrode and it may be that there is a preferred or ideal field gradient at the point of the electrode which can be achieved by adjustment of the voltage to suit the sharpness of the part of the electrode at which the denaturation takes place. Optionally, one can conduct the denaturation using a constant current supply rather than a regulated voltage and this may serve to compensate for variations in the geometrical set-up of the electrodes between different denaturation operations.

However, a further preferred electrode arrangement comprises a pair of parallel metal (e.g. platinum) plates. These are preferably spaced by up to 1.0 mm, e.g. from 0.3 to 0.5 mm.

Where a constant current regime is employed, it will generally be preferable to use a current of from 80 to 160 μA, e.g. about 100 to 125 μA. It should be borne in mind however that higher currents are desirable with increasing ionic strength of buffer used. The currents quoted are those for DNA in water.

As described in WO92/04470, one may employ a promoter compound such as methyl viologen to produce more rapid denaturation. Other promoters are described in WO93/15224, i.e. multivalent cations such as magnesium. Other multivalent cations which are effective and which can be used include lanthanum (La^{3+}). The cations used as the promoters may include inorganic cations complexed with inorganic or organic ligands, e.g. $Pt(NH_3)_6^{4+}$ and $Cr(NH_3)_6^{2+}$.

The promoter may be any inorganic or organic molecule which increases the rate or extent of denaturation of the double helix. It should be soluble in the chosen reaction medium. It preferably does not affect or interfere with DNA or other materials such as enzymes or oligonucleotide probes which may be present in the solution. Alternatively, the promoter may be immobilised to or included in material from which the electrode is constructed.

The promoter may be a water-soluble compound of the bipyridyl series, especially a viologen such as methylviologen or a salt thereof. Whilst the mechanism of operation of such promoters is presently not known with certainty, it is believed that the positively charged viologen molecules interact between the negatively charged nucleic acid such as DNA and the negatively charged cathode to reduce electrostatic repulsion therebetween and hence to promote the approach of the DNA to the electrode surface where the electrical field is at its strongest. Accordingly, we prefer to employ as promoters compounds having spaced positively charged centres, e.g. bipolar positively charged compounds. Preferably the spacing between the positively charged centres is similar to

that in viologens. Other suitable viologens include ethyl-viologen, isopropyl viologen and benzyl viologen.

Optionally, the process may be conducted using a three electrode system of the kind described in WO92/04470 but generally it is preferred that the volume of solution employed according to this invention is small e.g. 1 ml or less, preferably very small e.g. 100 μl or less, e.g about 25 μl to 50 μl or less. When using very small reaction volumes of this kind, it will generally not be practical to use a three electrode system.

The process may be carried out at ambient temperatures or if desired at temperatures up to adjacent the pre-melting temperature of the nucleic acid. The process may be carried out at a pH of from 3 to 10, conveniently about 7. Generally, more rapid denaturation is obtained at lower pH. For some purposes therefore a pH somewhat below neutral e.g. about pH 5.5 may be preferred. The nucleic acid may be dissolved in an aqueous solution containing a buffer whose nature and ionic strength are such as not to interfere with the strand separation process.

Preferably, the solution contains a buffer at a concentration at least 10 mM, e.g. about 25 mM. Optionally, the solution may contain further salts such as magnesium chloride and sodium chloride. Preferably, the reaction is conducted in a buffer of the kind used in PCR or in LCR procedures.

Preferably therefore the ionic strength of the solution is above 20 mM, e.g. 25 to 50 mM.

The denaturing process according to the invention may be incorporated as a step in a number of more complex processes, e.g. procedures involving the analysis and/or the amplification of nucleic acid. Some examples of such processes are described below.

We have found that by virtue of the superior cell design described in WO95/25177 and herein, in combination with the voltage regime described herein it is possible to achieve denaturation much more quickly than before.

Amplification procedures within the invention include both PCR and LCR.

In such a polymerase mediated replication procedure, e.g. a polymerase chain reaction procedure, it may not be necessary in all cases to carry out denaturation to the point of producing wholly single-stranded molecules of nucleic acid. It may be sufficient to produce a sufficient local and/or temporary weakening or separation of the double helix in the primer hybridisation site to allow the primer to bind to its target. Once the primer is in position on a first of the target strands, rehybridisation of the target strands in the primer region will be prevented and the other target strand may be progressively displaced by extension of the primer or by further temporary weakening or separation processes.

Amplification processes according to the invention may be carried out over more than 1 cycle of the electrical profile, e.g. up to 5 to 10 cycles or more, e.g. up to 20 or 30 cycles. In the amplification process the hybridisation step is preferably carried out using two primers which are complementary to different strands of the nucleic acid.

The denaturation to obtain the extension products as well as the original denaturing of the target nucleic acid is preferably carried out by applying to the solution of the nucleic acid the voltages from the electrodes.

The process may be a standard or classical PCR process for amplifying at least one specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids wherein each nucleic acid consists of two separate complementary strands, of equal or unequal length, which process comprises:

(a) treating the strands with two oligonucleotide primers, for each different specific sequence being applied, under conditions such that for each different sequence being amplified an extension product of each primer is synthesised which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to different strands of each specific sequence such that the extension product

synthesised from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) separating the primer extension products from the templates on which they were synthesised to produce single-stranded molecules by applying the voltages from the electrode to the reaction mixture; and

(c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesised using each of the single strands produced in step (b) as a template.

Alternatively, the process may be any variant of the classical or standard PCR process, e.g. the so-called "inverted" or "inverse" PCR process or the "anchored" PCR process.

The invention therefore includes an amplification process as described above in which a primer is hybridised to a circular nucleic acid and is extended to form a duplex which is denatured by the denaturing process of the invention, the amplification process optionally being repeated through one or more additional cycles.

The process of the invention is applicable to the ligase chain reaction in a process for amplifying a target nucleic acid comprising the steps of:

(a) providing nucleic acid of a sample as single-stranded nucleic acid;

(b) providing in the sample at least four nucleic acid probes, wherein:

i) the first and second said probes are primary probes, and the third and fourth of said probes are secondary nucleic acid probes;

ii) the first probe is a single strand capable of hybridising to a first segment of a primary strand of the target nucleic acid;

iii) the second probe is a single strand capable of hybridising to a second segment of said primary strand of the target nucleic acid;

iv) the 5' end of the first segment of said primary strand of the target is positioned relative to the 3' end of the second segment of said primary strand of the target to enable joining of the 3' end of the first probe to the 5' end of the second probe, when said probes are hybridised to said primary strand of said target nucleic acid;

v) the third probe is capable of hybridising to the first probe; and

iv) the fourth probe is capable of hybridising to the second probe; and

(c) repeatedly or continuously:

i) hybridising said probes with nucleic acid in said sample;

ii) ligating hybridised probes to form reorganised fused probe sequences; and

iii) denaturing DNA in said sample by applying said voltages as described to the reaction mixture.

The electrochemical DNA amplification technique can be used analytically to detect and analyse a very small sample of DNA e.g a single copy gene in an animal cell or a single cell of a bacterium.

The temperature at which the process is carried out may be chosen to suit whichever enzyme is used. Thus where Taq is used as polymerase, a temperature of 55 to 68°C is preferred. If Klenow polymerase is used, ambient temperature will be suitable. For Vent polymerase one preferably uses from 50 to 72°C. It may be desirable to employ known protein stabilisation techniques to avoid electrical damage to the polymerase, especially where a mesophilic polymerase is used.

The invention includes a process for detecting the presence or absence of a predetermined nucleic acid sequence

in a sample which comprises: denaturing a sample double-stranded nucleic acid by means of said voltages applied to the sample in a solution; hybridising the denatured nucleic acid with an oligonucleotide probe for the sequence; and determining whether the said hybridisation has occurred.

Thus, the invented process has application in DNA and RNA hybridisation where a specific gene sequence is to be identified e.g. specific to a particular organism or specific to a particular hereditary disease of which sickle cell anaemia is an example. To detect a specific sequence it is first necessary to prepare a sample of DNA, preferably of purified DNA, means for which are known, which is in native double-stranded form. It is then necessary to convert the double-stranded DNA to single-stranded form before a hybridisation step with a labelled nucleotide probe which has a complementary sequence to the DNA sample can take place. The denaturation process of the invention can be used for this purpose in a preferred manner by carrying out the following steps:

- denaturing a sample of DNA by applying voltage cycles by means of electrodes to the sample DNA with optionally a promoter in solution or bound to or part of the structure of the electrode;
- hybridising the denatured DNA with a directly labelled or indirectly labelled nucleotide probe complementary to the sequence of interest; and
- determining whether the hybridisation has occurred, which determination may be by detecting the presence of the probe, the probe being directly radio-labelled, fluorescent labelled, chemiluminescent labelled or enzyme-labelled or being an indirectly labelled probe which carries biotin for example to which a labelled avidin or avidin type molecule can be bound later.

In a typical DNA probe assay it is customary to immobilise the sample DNA to a membrane surface which may be composed of neutral or charged nylon or nitrocellulose. The

immobilisation is achieved by charge interactions or by baking the membrane containing DNA in an oven. The sample DNA can be heated to high temperature to ensure conversion to single-stranded form before binding to the membrane or it can be treated with alkali once on the membrane to ensure conversion to the single-stranded form. The disadvantages of such methods are:

- heating to high temperature to create single-stranded DNA can cause damage to the sample DNA itself.
- the use of alkali requires an additional step of neutralisation before hybridisation with the labelled probe can take place.

One improved method for carrying out DNA probe hybridisation assays is the so-called "sandwich" technique where a specific oligonucleotide is immobilised on a surface. The surface having the specific oligonucleotide thereon is then hybridised with a solution containing the target DNA in a single-stranded form, after which a second labelled oligonucleotide is then added which also hybridises to the target DNA. The surface is then washed to remove unbound labelled oligonucleotide, after which any label which has become bound to target DNA on the surface can be detected later.

This procedure can be simplified by using the denaturing process of the invention to denature the double-stranded DNA into the required single-stranded DNA. The working electrode, counter-electrode and optionally a reference electrode and/or a promoter can be incorporated into a test surface or a well in which the DNA probe assay is to be carried out. The DNA sample and oligonucleotide probes can then be added and the voltage applied to denature the DNA. The resulting single-stranded DNA is hybridised with the specific oligonuclectide immobilised on the surface after which the remaining stages of a sandwich assay are carried out. All the above steps can take place without a need for high temperatures or addition of alkali reagents as in the conventional process.

The invention will be further described and illustrated with reference to the accompanying drawings in which:

Figure 1 is a cross-sectional view through a first example of a cell for use in accordance with the invention;

Figure 2 is a cross-section through an alternative form of cell for use in the invention;

Figure 3 shows an exploded perspective view of a third form of cell for use in the invention;

Figure 4 is a side view of the cell of Figure 3;

Figure 5 is a plan view of the cell of Figure 3;

Figure 6 shows six voltage cycles for use with the cells of Figure 1 and Figure 2; and

Figures 7 to 15 show gels produced in the examples.

The cell illustrated in Figure 1 comprises a graphite block 10, containing a 4 mm diameter well 12 on its upper surface, constituting a first electrode. A second electrode formed from a 2 mm diameter graphite rod 14 having a tapering end portion 16 is pressed into the well through an insulating collar of plastics material 18. The rod is adjusted downwards in the well until it forms a short circuit and is then lifted back by as little as possible to open the circuit again. The capacity for liquid for the cell is approximately 25 μl.

Although the rod electrode shown in Figure 1 is blunt ended, it provides a sharp edge between its flat end and its tapering frustoconical surface. An optional alternative configuration is for the rod to be sharpened to a point. This may be achieved by using a conventional pencil sharpener. The resulting electrode may be further smoothed using a blade or abrasive. Using such a pointed electrode, the capacity for liquid of the well is increased to 40 μl.

The cell shown in Figure 2 comprises a graphite block 20 fabricated from 6B pencil graphite containing a 3mm diameter well 22, which is 15mm deep, having at its upper end an enlarged diameter mouth portion 24. A probe electrode 26 is formed from a 2B to 2H, e.g. HB pencil graphite and is 2mm in

diameter. It is supported in the well 22 by a sleeve 28 of PTFE in a base in which it is tightly received. A pair of O-rings 30 received in grooves in the sleeve 28 provide a snug fit for the sleeve 28 in the mouth portion 24 of the block 20. The precise height of the probe electrode in the well can be set by protruding by the desired extent fromt he lower end of the sleeve 28. The end of the probe electrode is flat and not tapered.

Multiple processes according to the invention may be carried out simultaneously in apparatus containing a multiplicity of sample receiving wells each provided with a respective electrode pair, one electrode in each case optionally being the well itself. In a preferred form for such apparatus a block containing the wells has a liftable lid with electrodes depending therefrom into the wells. Each well may have a pair of electrodes on the lid in various possible electrode conformations such as parallel rods, parallel plates, optionally of mesh, or coaxial hollow cylinders, again optionally of mesh. Alternatively, single electrodes may be provided on the lid for each well and the block containing the wells may be conductive and may serve as a common second electrode. The block of wells may also contain respective electrodes for each well.

The lid may comprise a flat plate portion bearing the electrode or electrodes for each well and a separate backing member bearing electrical connections and circuitry which connects up the electrodes when the two parts are assembled. A single electrical supply to the unit may be split by said circuitry and supplied in a controlled manner to the electrodes such that each electrode is controlled in voltage. The plate portion carrying the electrode array may thereby be replaceable without replacement of the control circuitry and may be made disposable. The plate portion and the backing member may be aligned with one another on assembly by locating pins and apertures and may similarly by aligned with the block containing the wells, which also may be disposable.

The cell shown in Figures 3 to 5 comprises a sandwich formed between a pair of opposed glass plates 30, 32 outside a pair of opposed platinum foil electrodes 34, 36 which lie either side of a spacer sheet 38 of a silicone elastomer (silastic™). Each electrode is provided with a connecting tag 42, 44 by which electrical connection is made to it. A chamber 40 is formed in the silastic™ sheet. The chamber consists of a circular cut-out of diameter approximately 10 mm and a lead-in channel extending to the edge of the sheet. The silastic™ sheet is approximately 400 μm in thickness.

Optionally two similar cavities 40 (or more) may be provided. The provision of two cavities in the silastic™ sheet enables the conduct of two reactions according to the invention, or one such reaction with a control, simultaneously.

Thus, it can be seen that illustrated electrochemical cell comprises two opposing planar platinum electrodes. The electrodes are separated by a sheet of deformable insulating material (in this instance a silicone elastomer) which forms a seal against liquid loss, and which is cut to form the electrode chamber. The electrodes are backed by flat plates, and the whole is clamped together between aluminium blocks (not shown) compressing the elastomer sheet from a free state thickness of about 500 μm. The potential difference between the electrodes and the polarity reversal pattern over time are set on a PC, which controls a power supply. The electrode assemblies are maintained at the operating temperature (suitably 55°C) by standing them on a heating block of appropriate temperature.

Voltage cycle No. 1 in Figure 6 consists of

a seconds at a positive voltage (suitably 1.6V as shown),

b seconds at a negative voltage,

c seconds at substantially zero voltage,

b′ seconds at the negative voltage,

a′ seconds at the positive voltage and

c seconds at substantially zero voltage,

repeated in cycles. Time values found suitable include:

a = b′ = 25s, b = a′ = 5s, c = 25 to 45s.

Voltage cycle No. 2 is the first half cycle of cycle No. 1. This will provide amplification. Use of the second half cycle instead by itself does not appear to provide amplification although it does contribute to the overall good performance of cycle No. 1.

Cycle No. 3 comprises six repeats of:

a seconds of positive voltage and

b seconds of negative voltage, followed by

b′ seconds of negative voltage and

c seconds of substantially zero voltage

in which suitable time values are

a = 5s, b = 0.2s, b′ = 5s, c = 45s.

In each case illustrated 12 cycles of the illustrated profile are generally sufficient in an amplification.

Voltage cycle No. 4 comprises periods of positive voltage in which the electrode 14 is made positive with respect to the well for a time a seconds followed by periods of zero voltage of duration c seconds followed by periods of negative voltage of duration b seconds. A further period of zero voltage of c seconds duration ends the cycle which is then repeated. The well is kept at earth in all the illustrated cycles.

In voltage cycle No. 5, the first of the zero voltage periods is omitted and one goes directly from the positive voltage of a seconds duration to the negative voltage of b seconds duration and this is followed by a period of c seconds at zero voltage.

In voltage cycle No. 6, a further period of b seconds of negative voltage is interposed between the period of c seconds of zero voltage and the start of the next cycle. Generally it may be said that the order in which the periods of positive, zero and negative voltage occur and the number of times they occur in each cycle is largely immaterial provided

that all three periods do occur, but there is a preference for starting with a positive voltage.

In voltage cycle No. 7, there is a positive voltage period a followed by a similar duration negative voltage period b. There is then a brief zero voltage period c followed by a return to negative voltage for b seconds and then to positive voltage for a seconds. This cycle is succeeded by a much longer zero voltage period c′ befor being repeated.

Strictly speaking, it is not necessary that the application of the voltages be cyclic so that periods of positive, zero and negative voltage occur in some repeated order. If these voltages are applied in random sequence a satisfactory result may be obtained but this is unnecessarily complicated in practice and it is preferable that the positive voltage periods occur at fixed intervals.

Preferred values for a and b in the illustrated voltage cycles 4 to 6 are from 5 to 20 seconds. Preferred values for c in each cycle are from 30 to 120 seconds.

The invention will be further illustrated by the following examples.

Example 1

Electrical PCR using Vent Polymerase.

Amplification of a 500 bp amplicon is carried out in the probe/well electrode system shown in Figure 1 or Figure 2 at 1.6 volts. The following mixture is placed in the well.

Electric PCR mix: i.e.
20 μl vent buffer
2 μl $MgSO_4$
32 μl primer #1 and #2
20 μl 1/20 dilution of 500 bp amplicon
1 μl Vent polymerase
85 μl dH_2O

Amplification was conducted using 10, 30 second pulses of 1.6 volts (positive and negative for alternate pulses), at

55°C with 2 minutes annealing and extension being allowed at zero volts after each positive and each negative pulse. Amplification was found to occur.

Example 2

The conditions of Example 1 were varied to the extent described below.

The agarose gel shown in Figure 7 illustrates amplification of a 500 bp amplicon with varying exposures to the electric field and annealing and extension times.

The lanes in Figure 7 are as follows:-

M Lambda Hind III marker.

O Control: no electrical pulses, held on ice.

OH Control: no electrical pulses, incubated at 55°C.

1-3 10 cycles of 1.6 volts positive for 30 seconds, 1.6 V negative for 30 seconds, and 2 minutes zero voltage. Each lane represents a different carbon well electrode.

The arrow marks the level at which the amplicon runs on the gel.

Lanes 1 and 2 show significant amplification compared to the controls, non specific amplification, probably of contaminants, has also occurred.

Example 3

The conditions of Example 1 were varied to the extent described below.

Figure 8 is an agarose gel showing the results.

The lanes are as follows:-

M Lambda Hind III marker.

O Control: no electrical pulses, held on ice.

OH Control: no electrical pules, incubated at 55°C.

1-2 10 cycles, 1.6 Volts for 15 seconds positive, 15 seconds negative polarity followed by 2 minutes annealing and extension.

The amplicon runs at the level marked by the arrow.

Example 4

The conditions of Example 1 were varied to the extent described below.

Figure 9 is an agarose gel showing the results.

The lanes are as follows:-

O Control: no electrical pulses, held on ice.

OH Control: no electrical pules, incubated at 55°C.

1-6 15 seconds positive, 15 seconds negative polarity at 1.6 volts for 10 cycles with 1 minute annealing and extension time. Using six different carbon well electrodes

M Lambda Hind III marker.

Amplification occurred visibly in all cases (seen at the level marked by the arrow), controls are not visible due to the dilution factor. The annealing and extension time has been reduced by one minute, whilst still allowing amplification to occur. This results in a total time of 15 minutes for electrical PCR.

Example 5

Confirming the importance of reversing the polarity.

To confirm that reversing the polarity is important for amplification, electrical PCR was tested at 1.6 volts, using 10, 30 second positive pulses with 2 minutes annealing and extension time, using the standard ePCR reaction mix described in Example 1.

The conditions of Example 1 were varied to the extent described below.

Figure 10 is an agarose gel showing the results.

The lanes are as follows:-

M Lambda Hind III marker.

O Control: no electrical pulses, held on ice.

OH Control: no electrical pules, incubated at 55°C.

1-6 6 different cuvette well electrodes.

No amplification occurred under these conditions. These data confirm that reversing the polarity is highly beneficial for ePCR amplification.

Example 6

Electrical amplification of 2 ng/50 μl 500 bp amplicon.

The conditions of Example 1 were varied to the extent described below.

Figure 11 is an agarose gel showing the results.

The lanes are as follows:-

M Lambda Hind III marker.

O Control: no electrical pulses, held on ice.

OH Control: no electrical pules, incubated at 55°C.

1-3 1.6 volt pulses of 10 seconds positive, 10 seconds negative followed by 1 minute annealing and extension (a = repeated) using three different cuvette well electrodes.

4-6 As above, but the voltage was lowered to 1.4 volts (a = repeated).

Amplification has again occurred. The amount of amplified product is variable, probably due to variation between the electrodes used.

Example 7

Using the cell of Figure 2 and the electrical profile shown at (1) in Figure 3, electrical PCR was carried out using the following mixture:

Mix	μl
H_2O	234
10x Vent Buffer	80
125μM dNTP's	64
20μM primer 2	4
20μM primer 3	4
100mM $MgSO_4$	2
100ng/ml Template	10
2U/μl Vent	1

Vent buffer consists of 20 mM TrisHCl, 10 mM KCl, 10 mM$(NH_4)_2SO_4$ and 0.1% Triton.

In Figure 12, lanes 1-3 show the results obtained with different electrodes (all according to Figure 2) and lane 4 shows a thermal control. The amplicon is marked by the arrow.

Example 8

This example shows how fine tuning of the conditions can improve the amplification obtained as is true also when using conventional thermal PCR. Example 7 is repeated using different strengths of buffer, but with the concentration of $MgSO_4$ maintained constant at 4.5 mM. The lanes in Figure 13 are as follows:-

Lanes	
1-3	1 x buffer
4-6	2 x buffer
7-9	3 x buffer
10-11	3.5 x buffer
12-13	4 x buffer
14-15	5 x buffer

The best results are obtained at 2 to 3 x buffer.

Example 9

The following reaction mixture was subjected to electrical PCR in the cell shown in Figures 3 to 5 using the voltage profile shown as No. 7 in Figure 6. The voltage periods were

a = 10 seconds, b = 10 seconds,

c = 1 second, c' = 50 seconds.

A voltage of 0.5 - 0.6 5V was applied during both the positive and negative voltage periods. Twenty cycles of the voltage profile were used.

Reaction Mix

10mM Ches buffer, pH 7.5

0.1% Triton X-100 and 3mM $MgSO_4$,

200 μM of each dNTP,

0.2 μM if each primer to give a 375bp fragment,

1 ng pUC linearised plasmid as starting material ($1x10^{-9}$g),

0.5U Vent polymerase,

Thermal profile (in control runs): 25 cycles of:

94°C-45 sec

54°C-20 sec

72°C-25 sec

followed by 1 cycle of: 72°C-2 min.

55°C constant on hot block

Electrical amplification was achieved at a potential of 0.5V (Figure 14). This voltage may not cause full denaturation, but there is clearly a sufficient degree of strand separation occurring to allow primer binding and extension. Also, it was found that consistency was improved by increasing the concentration of Ches to 10mM, which although not optimal for denaturation may have the effect of stabilising the reaction components.

The identity of the electrical amplification bands generated was confirmed by the following tests:

- initial visual screening on an ethidium bromide-stained gel,
- extraction of the sample with phenol-chloroform-ISA and precipitation from the aqueous phase with ethanol and re-running on a gel,
- detection of the biotinylated amplicon in the Dig-based assay
- the non-appearance of the band (in an agarose gel) following treatment of the electrically-generated amplicon with DNase (Figure 15).

Table 1 illustrates the results of a Dig assay, which detects biotinylated amplicons of the desired sequence. The strongest signals were produced from the amplicons which gave bands in gels of highest intensities. In some cases, the biotin test readings were approximately 50% of those from the thermal control product.

TABLE 1

Biotin-Dig Assay Results		
	375 bp band on gel	Absorbance at 405nm
Electrical amplification product, unpurified	+	0.40
Electrical amplification product, purified by phenol extraction and ethanol precipitation	+	0.50
Electrical amplification mix, no electric field cycling	-	0.05
Thermal PCR product, purified	+	1.20
Thermal PCR negative control (no polymerase)	-	0.06
Test reagent blank	-	0.05

The data presented here demonstrate using electrical denaturation to achieve isothermal amplification of a 375bp sequence from 2686bp linearised pUC DNA.

Denaturation has been found to be sensitive to the condition of the electrode surfaces, and electrical amplification even more so. Recommended electrode preparation techniques include:

1. Electrode cleaned using Brasso metal polish and a cotton bud. Before the polish dries wash with detergent and rinse under the hot tap till the Pt surface shows hydrobobicity, rinse well with dH_2O. Flick residual water off and allow to dry.

2. The electrodes are further cleaned by an electrochemical method. Sweeping between + and -2.4V at a ramp rate of 0.02V/s. This is performed for at least 1 hr. A faster ramp rate can also be used of 0.1V/s,

3. Check that electrical denaturation of 0.3μg/ml linear pUC18 in 5mM CHES pH7.6 occurs at 0.8V. This is a requirement before ePCR can be attempted. The denaturation priming generally requires a few cycles at 1.8V in the presence of pUC then denaturation cycles from 1.8V down to 0.8V in 0.2V steps.

Generally a suitable PCR reaction mix is:-

1/8 x Thermopol Buffer
5mM CHES pH7.6
0.075% Triton X-100
0.6μM Primer#2
0.4μM Primer#1
200μM dNTP's
~9ng Lin pUC18
3mM $MgSO_4$

Without wishing to be bound by any theory, we believe it is probable that reversing the polarity during each cycle may be forcing the DNA away from the electrode surface back into solution where it mixes with the other PCR components allowing annealing and extension to proceed.

Whilst the invention has been described above in terms of the preferred embodiments illustrated by the examples, many modifications and variations thereof are possible within the scope of the invention.

CLAIMS

1. A process for denaturing double-stranded nucleic acid which comprises subjecting a said nucleic acid to a voltage applied between electrodes under conditions such as to convert at least a portion of said nucleic acid to a wholly or partially single stranded form, wherein said voltage is applied such that there are, in any order, periods of application of voltage with a first polarity, periods of application of voltage with the opposite polarity to said first polarity and periods of substantially reduced applied voltage.

2. A process as claimed in Claim 1, wherein said cycles are from 1 second to 5 minutes in length.

3. A process as claimed in Claim 2, wherein the periods during which said voltage is applied with a first polarity and said periods during which said voltage is applied with a second polarity are each independently of from 0.5 seconds to 1 minute.

4. A process as claimed in Claim 2 or Claim 3, wherein the periods during which said voltage is substantially zero are each independently from 0.5 seconds to 3 minutes.

5. A process as claimed in any preceding claim, wherein said electrodes approach to within 1.5mm of one another in said solution.

6. A process as claimed in Claim 5, wherein the electrodes approach to within 1 mm of one another in said solution.

7. A process as claimed in Claim 6, wherein the electrodes approach to within 0.5 mm of one another in said solution.

8. A process as claimed in any preceding claim, wherein a voltage of from 0.5 to 3 volts is applied between said electrodes.

9. A process as claimed in Claim 8, wherein a voltage of from 1.5 to 2.5 volts is applied between said electrodes.

10. A process as claimed in any preceding claim, wherein the solution contains a promoter which assists said denaturation.

11. A process as claimed in Claim 10, wherein said promoter is methyl viologen or a salt thereof, or is a multivalent inorganic cation.

12. A process as claimed in Claim 11, wherein said promoter is magnesium or lanthanum ions.

13. A process as claimed in any preceding claim, wherein where the electrodes most closely approach one another, one or both of the electrodes is pointed.

14. A process of amplifying a target sequence of nucleic acid comprising hybridisation, replication and denaturation of nucleic acid wherein said denaturation is conducted by subjecting a solution containing said nucleic acid to a voltage applied between electrodes under conditions such as to convert at least a portion of said nucleic acid to a wholly or partially single stranded form in the solution said voltage being applied such that there are, in any order, periods of application of voltage with a first polarity, periods of application of voltage with the opposite polarity to said first polarity and periods of substantially zero applied voltage.

15. A process of amplifying a target sequence of nucleic acid as claimed in claim 14, wherein said electrodes approach to within 1.5mm of one another in said solution.

16. A process as claimed in Claim 14 or Claim 15, wherein said cycles are from 1 second to 5 minutes in length.

17. A process as claimed in Claim 14, wherein the periods during which said voltage is applied with a first polarity and said periods during which said voltage is applied with a second plurality are each independently of rom 0.5 seconds to 1 minute.

18. A process as claimed in Claim 16 or Claim 17, wherein the periods during which said voltage is substantially zero are each independently from 0.5 seconds to 3 minutes.

19. A process of amplifying a target sequence as claimed in any one of claims 14 to 18, which is a PCR or LCR amplification.

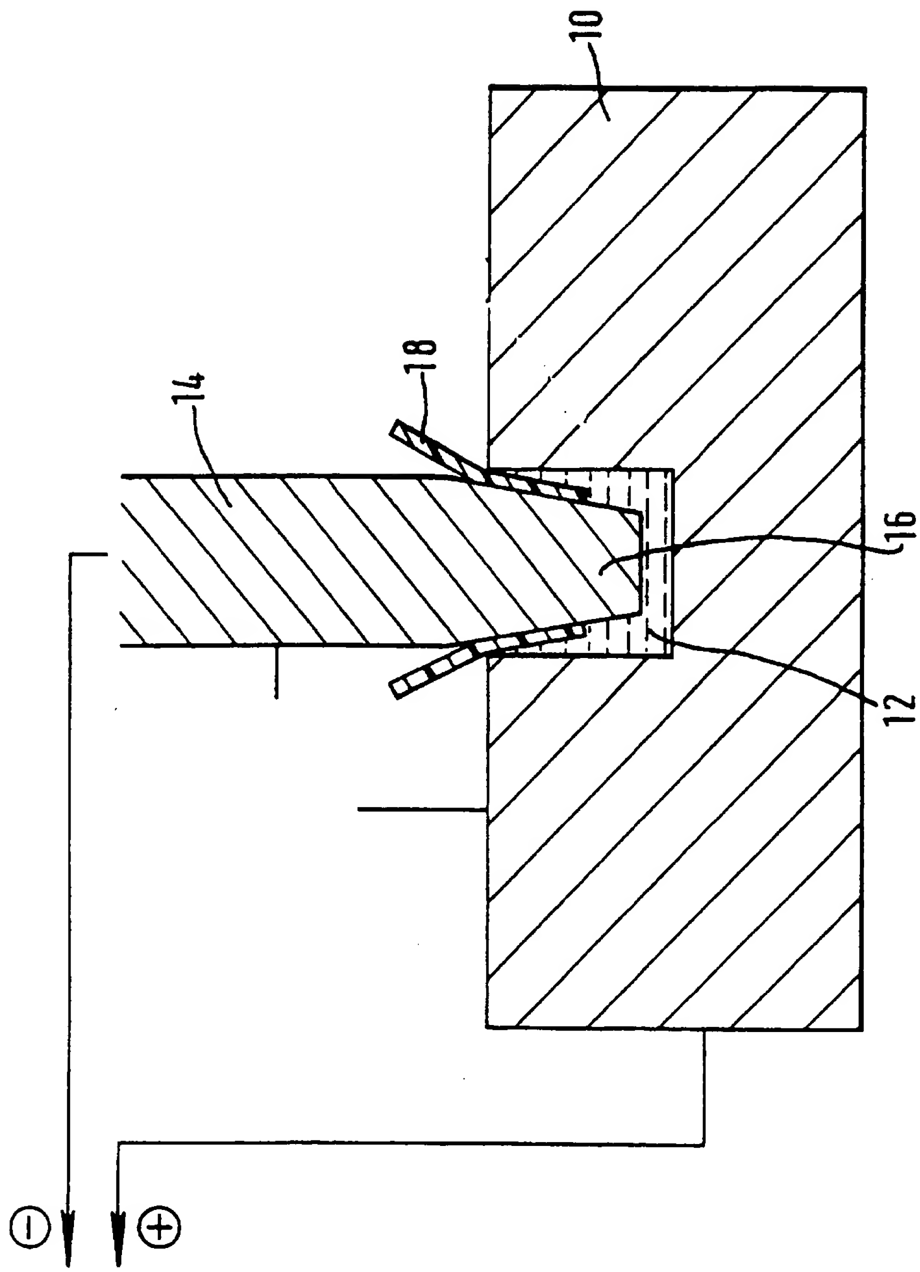


FIG 1

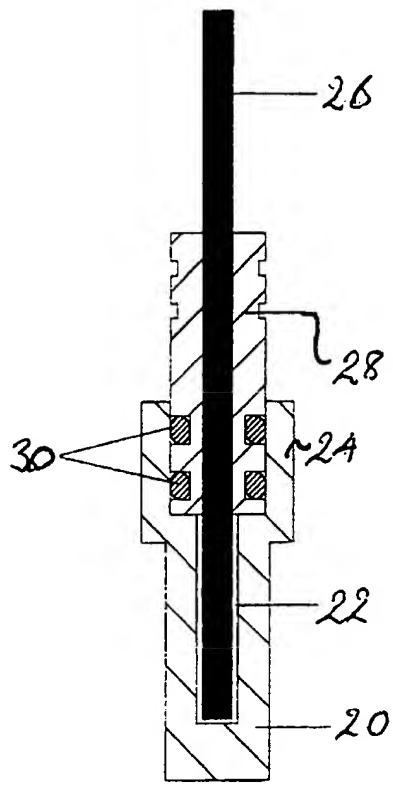


FIG 2

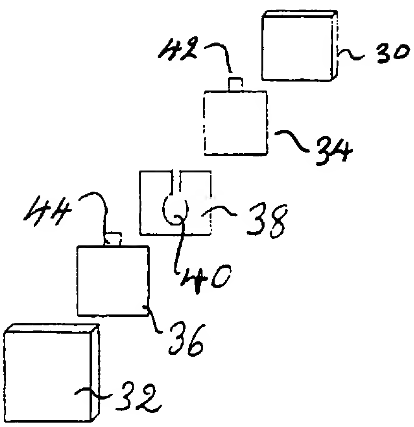


Fig. 3

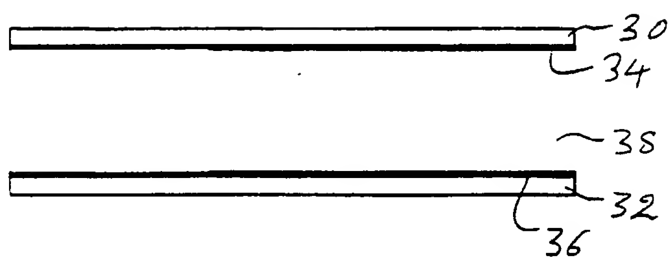
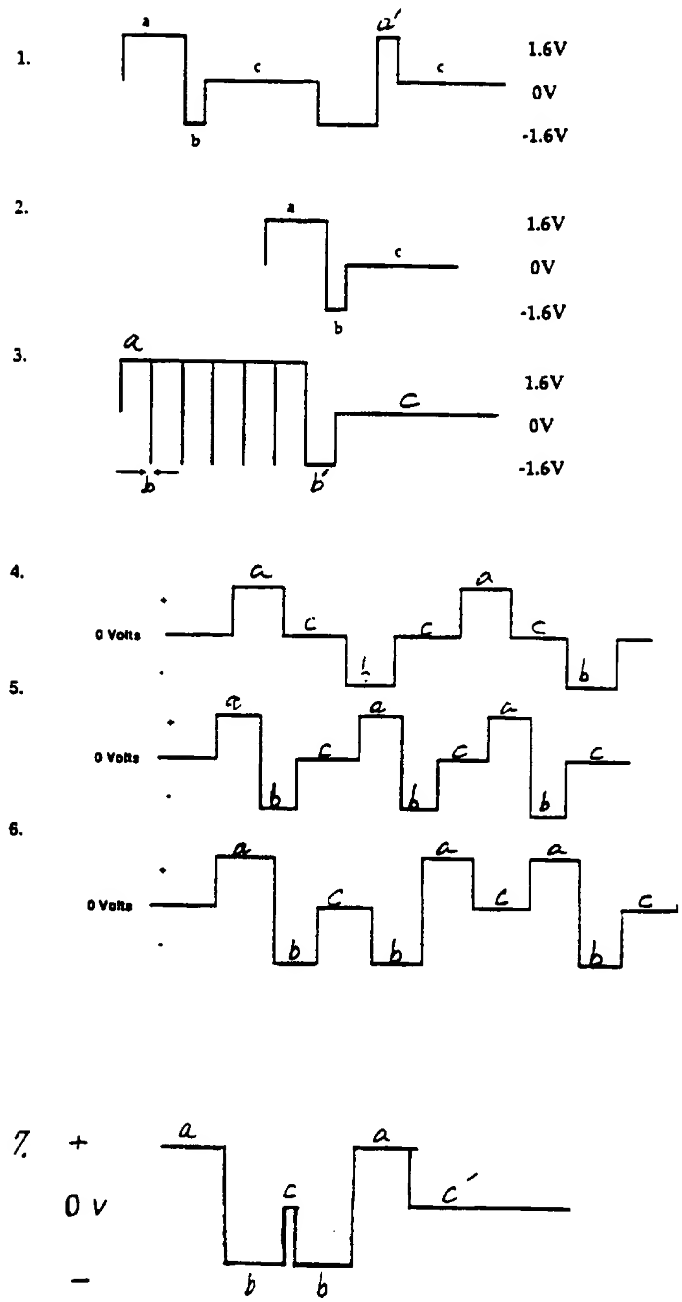


Fig. 4

Fig. 5

FIG.6


1.
a
a'
c
c
b
1.6V
0V
-1.6V
2.
a
c
b
1.6V
0V
-1.6V
3.
a
c
b
b'
1.6V
0V
-1.6V
4.
0 Volts
a
c
c
a
c
b
5.
0 Volts
a
b
c
a
b
c
a
b
c
6.
0 Volts
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b
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a
c
a
b
c
7.
+
0 v
−
a
b
c
b
a
c'

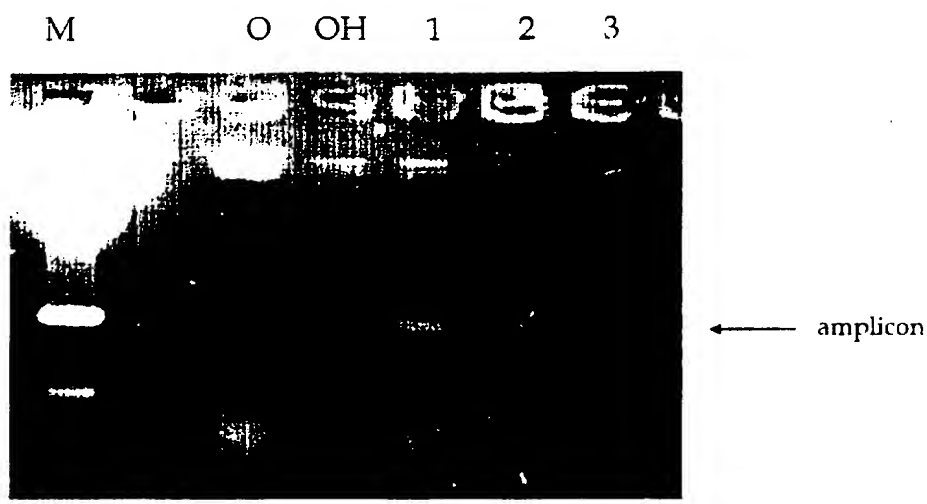

FIG 7

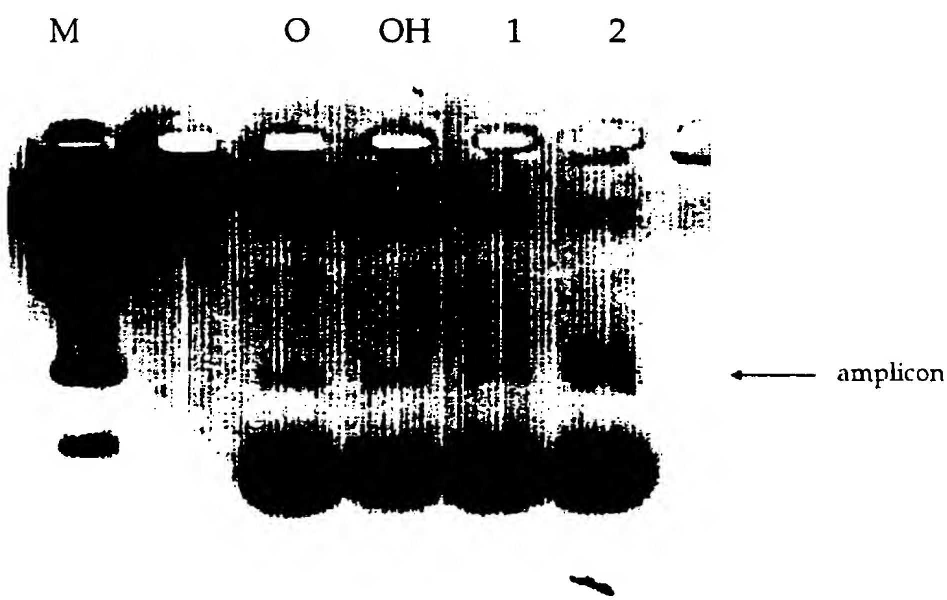

FIG 8

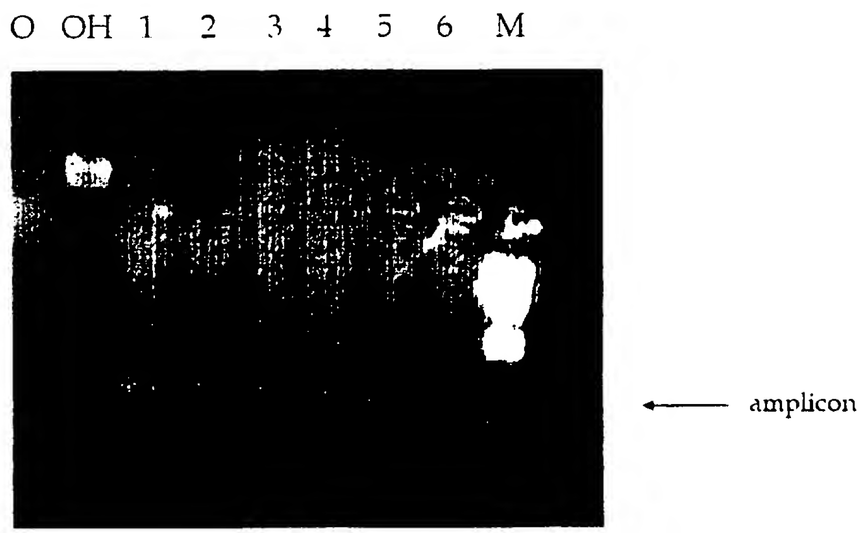

FIG 9

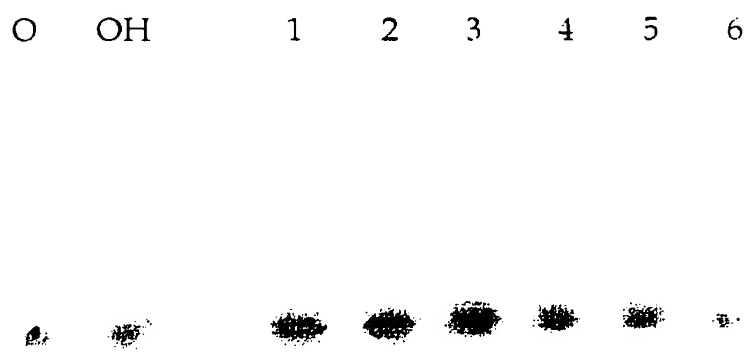

FIG 10

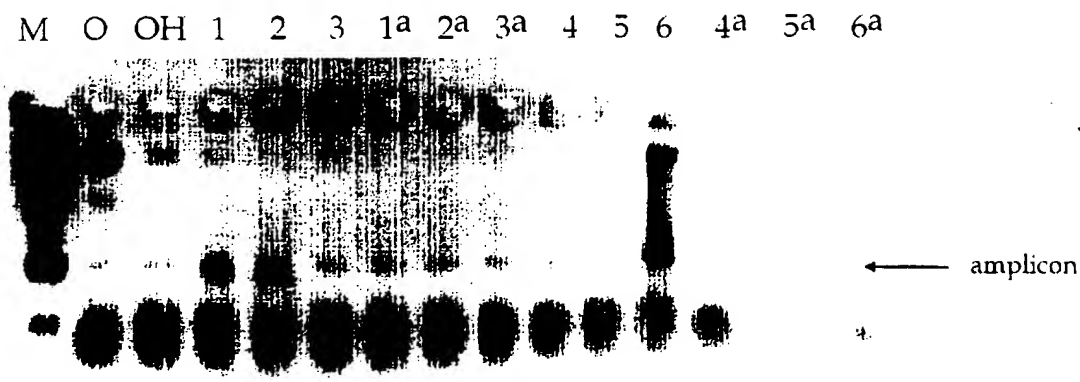

FIG 11

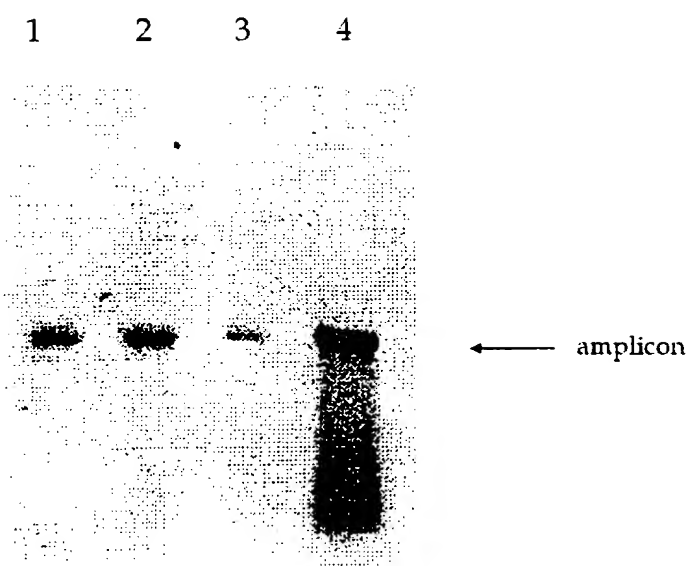

FIG 12

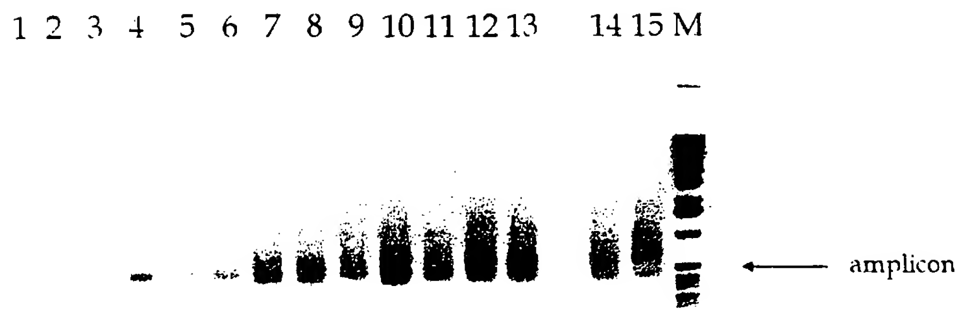

FIG 13

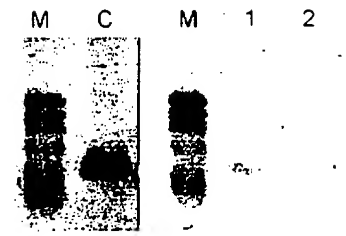

Fig. 14

Lane

M	ΦX174 DNA - *Hae* III digest
C	Thermal control. 375bp amplicon
1	Electrical amplification product following 20 cycles of electrical profile
2	Electrical amplification product following 20 cycles of electrical profile

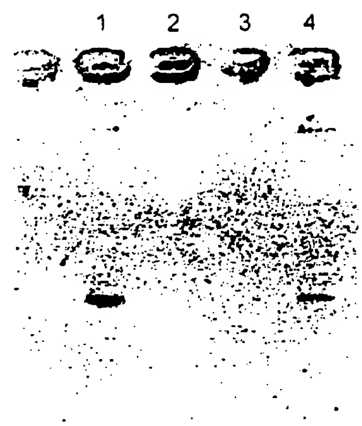

Fig. 15

Lane

1	Thermal control. untreated
2	Thermal control. treated with DNase
3	Electrical amplicon treated with DNase
4	Electrical amplicon (pooled)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/GB 97/01764

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 25177 A (SCIENT GENERICS LTD ;PURVIS DUNCAN ROSS (GB)) 21 September 1995 cited in the application see the whole document ---	1-19
A	WO 92 04470 A (SCIENT GENERICS LTD) 19 March 1992 cited in the application see the whole document ---	1-19
A	WO 93 15224 A (SCIENT GENERICS LTD) 5 August 1993 cited in the application see the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 1997	11.11.97

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01764

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C.J. STANLEY ET AL.: "Amperometric enzyme-amplified immunoassays" J. IMMUNOL. METHODS, vol. 112, no. 2, 1988, ELSEVIER, AMSTERDAM NL, pages 153-161, XP002043865 cited in the application see the whole document ---	1-19
P,A	WO 97 08293 A (SCIENT GENERICS LTD ;MARTIN SOPHIE ELIZABETH VICTOR (GB); BERGMANN) 6 March 1997 see the whole document -----	1-19

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INTERNATIONAL SEARCH REPORT

ɩormation on patent family members

Intern ıal Application No
PCT/GB 97/01764

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